Cloning of Binding Domain of *Clostridium botulinum* Toxin Type A in *Escherichia coli*

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Summary

Neurotoxin of C*lostridium botulinum* is one of the most potent known toxins in nature, which can cause an often fatal food poisoning. However, recently the use of binding domain of the toxin has also been proposed as a candidate vaccine. In this study coding region for this domain was amplified from genomic DNA of *Clostridium botulinum* type A using a pair of primers containing *Bam*HI and *Hind*III. The amplified 1.3kb fragment corresponding to the gene of interest was cloned in pUC18 after verification with different restriction enzymes. The construct was then transformed into competent *Escherichia coli* DH5α and the recombinant clone was selected on plates containing ampicillin. The plasmid purified from these clones was used for sequencing by dideoxy chain termination method. The sequence thus obtained was verified by comparison with published sequence, which showed the authencity of the PCR product. This fragment was subcloned in an IPTG inducible expression vector pQE30 using *E.coli* M15 as a host and expression was analyzed by RT-PCR.

***Key words*:** Botulinum, neurotoxin, binding domain, expression, *Escherichia coli*

Introduction

Clostridium botulinum an anaerobic spore-forming gram positive bacillus, produces several highly toxic types of botulism neurotoxins classified A through G (Sugiyama 1980). The most common mechanism of botulinum poisoning is through oral ingestion of food contaminated with a 900kDa complex constituting of a toxic (neurotoxin) and a non-toxic component (hemagglutinin) (Chen 1997, Dasgupta 1968). Although ingested spores of the bacterium may also colonize and produce

toxin in the intestinal tract of infants, resulting in infant botulism (Tacket & Rogawski 1989). The toxin is synthesized as a 150kDa precursor which (with the exception of type E) is proteolytically cleaved into two subunits designated A- and BC-fragments corresponding to the light (50kDa) and heavy (100kDa) chains, which remain covalently associated by a disulfide bond (Binz 1990). Toxification by botilinum type A (BoNT/A) is initiated by binding of the toxin molecules to specific ganglioside receptors of the presypnatic membrane, followed by receptor mediated endocytosis and finally inhibition of acetylcholin release at neuromuscular presynaptic nerve endings causing paralysis and death (Coffield 1997, Montecucco 1996).

The current prevalent toxoid vaccine for botulinum is composed of formaline inactivated holotoxin derived from serotypes A-E. Although effective, this vaccine is difficult to manufacture, since large quantities of toxin from each serotype must be produced and isolated and also extensive treatment with formalin is required to inactivate the toxin (Dertzbaugh & West 1996, LaPenotiere 1995). This method of vaccine production suffers from several major problems, leading to a search for better and cheaper vaccines, most notably through the use of DNA recombinant technology.

The gene for BoNT/A has been cloned and it has been shown that H-chain can be further cleaved to yield a non-toxic 50kDa C-terminal fragment (Hc) that is involved in binding to extracellular ecto-receptors (Montecucco 1996). This fragment is thought to contain the principal protective antigenic determinants, because immunization with it or with some of its sub-fragments can induce protective immunity in mice ((Dertzbaugh & West 1996, Clayton 1995). Furthermore, laboratory detection and identification of BoNT-producing clostridia on the basis of bacteriological characteristics are difficult and currently the most sensitive method of BoNT is the mouse bioassay. However, this technique is time consuming and requires handling of laboratory animals (Fach 1995) which might be overcome if sensitive and reliable immunological assays are developed. Therefore, in the present study, in view of above, the cloning and expression of the binding domain was undertaken, and in order to overcome the problem of low expression pQE30 was chosen in which a 6x-His tag is fused to the N-terminus of the protein. This strategy had not been used in previous studies.

Materials and Methods

Bacterial strains and plasmids. *Cl.botulinum* type A isolated from patient in Pasteur institute of Iran (subtype A strain p12) was used for isolation of binding domain of BoNT.

pUC18 vector (Pharmacia) together with *E.coli* strain DH5α [supE44 Δlac u169 (∅80 lacZΔ M15) hsdR17 recA1 endA1 gyrA96 thi *1relA1*] were used for cloning and maintenance of DNA fragment. For protein expression pQE -30 (QIAGEN) carrying strong bacteriophage T5 promoter and two lac operator and synthetic ribosomal binding site with 6xHis tag for detection and purification step was used. *E.coli* strain M15 [pREP4 (*Nal,Str,Rif,Lac-ara-gal mtl-f-recA+uvr+*)] was used as host strain .

DNA extraction. Genomic DNA was extracted using the method described by Robert *et al* (1987) and assayed on 0.8% agarose gel and evaluated using spectrophotometry. Alkaline lysis method (Birnboim & Doly 1979) was used for plasmid isolation.

Gene amplification. Oligonucleotid primers containing *Bam*HI and *Hind*III were designed according to the published data (Binz 1990) for Hc fragment with the following sequences:

Forward primer 5'-TAT GGA TCC ATT ATT AAT ACT TCT ATA TTG AA-3'

*Bam*HI

Reverse primer 5'-TAG TTT GAA AGC TTT TAC AGT GGC CTT T-3'

*Hind*III

Amplification was performed as follow:

95˚C	5min		hot start
94˚C	1min	|	
55˚C	1min	|	10 cycle
72˚C	1min	|	
94˚C	1min	|	
64˚C	1min	|	25 cycle
72˚C	1min	|	
72˚C	10min		Final extension

Primary amplification was performed using Taq DNA polymerase (Sinagen), but after optimization of PCR condition, in order to minimize mismatch incorporation VENT^{TM} DNA polymerase (New England Biolab) was used for final amplification.

Elution of DNA fragment from agarose gel. For elution of PCR fragments from agarose gel, the DEAE membrane (Schleicher & Schuell Co.) method was used (Sambrooke *et al* 1989).

Plasmid construction. The amplified product was digested with *Bam*HI and *Hind*III (Boheringer Mannheim), cloned in pUC18 (Pharmacia Biotech) and the construct was transformed into competent *E.coli* DH5α in LB Media (Difco) using standard methods (Sambrooke 1989). The presence of binding domain of BoNT/A coding region was confirmed by restriction enzyme analysis and the construct was used for sequencing by the method of Sanger (1977) on an automated sequencer (ALF system, Pharmacia). After sequence verification the gene encoding BoNT/A binding domain was subcloned in pQE-30 (QIAGEN) and transformed into competent *E.coli* M15. The clone containing the construct was selected on Luria Bertani (LB) media containing 100μg/ml ampicillin and 25μg/ml kanamycin, which were then used to express the recombinant protein.

Expression of the recombinant protein. Preliminary data on the expression of the Binding domain of BoNT/A at transcription level was obtained using RT-PCR. For this purpose *E.coli* M15 containing pQE30-binding domain were grown on LB broth supplemented with antibiotics (ampicillin 100μg/ml and kanamycin 25μg/ml) and incubated at 37˚C with 110rpm/min to early exponential phase ($OD_{600}\cong 0.6$). IPTG was added to a final concentration of 1mM and induction was continued for 4h. Cells from 5ml of the culture were harvested at 3000rpm and the pellet was used for total RNA extraction.

RT-PCR. Total RNA was extracted using TRIZOL (GibcoBRL) and cDNA was synthesized using random hexamers as primers and reverse transcriptase. The cDNA mixture was incubated at 65˚C for 5min followed by incubation at 37˚C for 1h. DNA was ethanol precipitated and then used for PCR using gene specific primers.

Results and Discussion

Diversity of neurotoxin types, low expression, special handling required for culture and purification of the toxin and the need for its extensive detoxification has made

the production of its vaccine and antibody difficult and expensive. Furthermore, the only reliable detection and typing method of the contaminating toxin is the mouse bioassay which is time consuming, making the need for research in this field an important health issue. It has recently been shown that the Hc fragment of the neurotoxin produced by *Cl.botulinum* type A is immunogenic and can produce immunity against this toxin in animals. Therefore, genomic DNA was extracted from this microorganism and the 1.3kb fragment corresponding to the coding region of the binding domain was amplified using PCR (Figure1).

Figure 1. *Genomic DNA extracted from Cl.Botulinum type A*

However, it was found that 2mM $MgCl_2$ and a two step amplification cycle, using two different annealing temperatures were required for efficient and specific amplification (Figure 2).

Lane 1:Amplification with Vent^{TM} DNA polymerase
Lane 2: Molecular weight marker.
Lane 3:Amplification with taq DNA polymerase

Figure 2. *PCR amplification of Hc fragment from genomic DNA of Cl.botulinum*

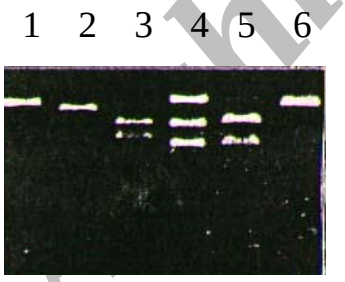
The detailed strategy used for cloning of the PCR fragment in both pUC18 and pQE-30 which was used for expressing the protein is shown in figure 3. Originally, it had been shown that attempts to express the native gene from *Cl.botulinum* type A in pTrc 99A in order to obtain the non-toxic binding domain of BoNT is unsuccessful and to achieve this aim either the gene should be assembled synthetically with codons preference of *E.coli* (Clayton 1995) or it has to be expressed in this host as fusion

protein (Michael *et al* 1995). Therefore the native gene was subcloned in pQE-30 which adds a 6xHis tag to the N-terminus of the expressed protein. Despite this strategy the expressed recombinant protein could not be detected in SDS-PAGE. Consequently, the integrity of the expression system at the construct level was examined by PCR and restriction enzyme analysis (Figure 4) and at transcription level by RT-PCR The results obtained showed that the native gene is fully transcribed and the mRNA transcript is still intact after the 4h period of induction (Figure 5).

However, since optimisation of conditions for culture, induction, extraction and purification of the recombinant protein are deemed essential (Hanning & Makrides 1998), therefore attempts for optimisation of these parameters were made as sumerised in table 1.

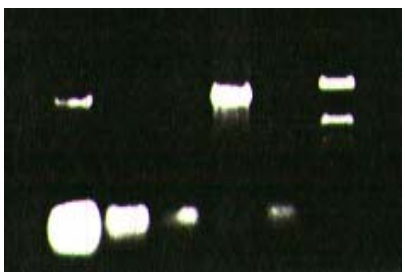
Table 1. *Conditions used for optimisation of expression of BoNT/A Hc fragment*

Culture media	**Ampicillin concentration**	**Time of induction (OD_{600})**	**IPTG concentration**	**Duration of induction**
LB	100µg/ml	0.5-1.1	0.5-1.0µg/ml	1-O/N
LB	200µg/ml	0.5-0.7	1.0µg/ml	4h
LB+ 0.2% Glucose	100µg/ml	0.5-0.7	1.0µg/ml	4h
LB+ 0.2% Glucose	200µg/ml	0.5-0.7	1.0µg/ml	4h



Lane 1: digest w/ Bgl II
Lane 2: digest w/ EcoRI
Lane 3: digest w/ Taq I
Lane 4: pUC-Taq marker
Lane 5: digest w/Hae III
Lane 6: undigest pcr product

Figure 4. *Restriction analysis of PCR product*



Lane 1 : concentrate cDNA
Lane 1 : concentrate cDNA
Lane 2 : 1/5 dilute cDNA
Lane 3 : 1/25 dilute cDNA
Lane 4 : positive control
Lane 5 : negative control
Lane 6 : M. W. marker

Figure 5. *Gel electrophoresis of RT-PCR product*

Figure 3. *Strategy used in this study for isolation and cloning of binding domain of Cl.botulinum type A*

The result of optimization experiment showed that addition of glucose to the medium has no profound effect on expression level. On the other hand our kinetic study of time of induction showed that the optimum OD for expression was early to

mid-log phase (0.5-0.7). The optimum concentration of IPTG was shown to be in the range of 0.5-1μg ,however the higher concentration indicated better result and as far as duration of induction was concerned the 4 period was found to be optimum time for expression. Furthermore, the use of two other expression vectors (pMAL and pGEX) are underway. Both vectors are part of an integrated system for the expression, affinity-purification of fusion proteins and contain the strong IPTG-inducable *tac* promoter. In pMAL system (New England BioLabs) the cloned gene is inserted into the vector downstream of *MAL E* gene which encodes the signal sequence of maltose-binding protein (MBP). This system has been successfully used for high level expression of different domains of BoNT/A neurotoxin.

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